Original Article

Sequence variations of the bovine prion protein gene (*PRNP*) in native Korean Hanwoo cattle

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Bovine spongiform encephalopathy (BSE) is one of the fatal neurodegenerative diseases known as transmissible spongiform encephalopathies (TSEs) caused by infectious prion proteins. Genetic variations correlated with susceptibility or resistance to TSE in humans and sheep have not been reported for bovine strains including those from Holstein, Jersey, and Japanese Black cattle. Here, we investigated bovine prion protein gene (*PRNP*) variations in Hanwoo cattle [*Bos* (*B.*) *taurus coreanae*], a native breed in Korea. We identified mutations and polymorphisms in the coding region of *PRNP*, determined their frequency, and evaluated their significance. We identified four synonymous polymorphisms and two non-synonymous mutations in *PRNP*, but found no novel polymorphisms. The sequence and number of octapeptide repeats were completely conserved, and the haplotype frequency of the coding region was similar to that of other *B. taurus* strains. When we examined the 23-bp and 12-bp insertion/deletion (indel) polymorphisms in the non-coding region of *PRNP*, Hanwoo cattle had a lower deletion allele and 23-bp del/12-bp del haplotype frequency than healthy and BSE-affected animals of other strains. Thus, Hanwoo are seemingly less susceptible to BSE than other strains due to the 23-bp and 12-bp indel polymorphisms.

Keywords: bovine spongiform encephalopathy, genetic polymorphisms, prion

Introduction

Bovine spongiform encephalopathy (BSE), better known to the public as mad cow disease, is one of the fatal neurodegenerative diseases that are collectively known as transmissible spongiform encephalopathies (TSEs). These also include Kuru and Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, and chronic wasting disease in deer [28]. Most TSEs are characterized by a long incubation period and a neuropathological feature of multifocal spongiform changes such as astrogliosis, neuronal loss, or an absence of inflammatory response. The causative agent of TSEs is an infectious prion protein (PrP^{Sc}), that is a structurally abnormal and protease-resistant isoform of the prion protein (PrP) [2]. PrP^{Sc} is derived from the endogenous cellular prion protein (PrP^{C}) that has undergone post-translational modification resulting in conformational changes [27,29]. PrP^{C} is known to play a role in copper metabolism [21,37] but its normal function(s) in cells are not well defined.

It is generally believed that BSE epidemics in cattle are caused by the unusual ingestion of meat and bone meal contaminated with PrP^{Sc} from BSE-affected cattle or scrapie-affected sheep [28]. BSE likely poses a threat not only to cattle but also to humans because variant CJD in humans is also reportedly caused by consuming meat or other products from BSE-affected cattle [3,35]. Therefore, many nations have focused their research efforts on BSE epidemics and implemented protocols to prevent BSE in cattle.

Recent studies on resistance or susceptibility to TSEs have focused on investigating the genetic structure of the prion protein gene (*PRNP*). In cattle, *PRNP* is located on chromosome 13 and the prion protein-coding region is positioned in exon 3 [31]. Polymorphisms of the prion protein-coding region can induce structural changes within the protein itself. Eventually, such structural changes in the prion protein reportedly result in the molecular pathology of TSEs. It has been reported that single nucleotide polymorphisms (SNPs) of *PRNP* have various effects on the susceptibility and incubation time of prion diseases in humans, mice, and sheep [1,22]. In sheep, polymorphisms

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in codons 136, 154, and 171 are correlated with susceptibility to scrapie and can thus be used to control disease incidence [8], whereas in humans, a polymorphism in codon 129 has a critical influence to variant CJD incidence [38]. To date, there are no known polymorphisms within the prion protein-coding region associated with BSE susceptibility in cattle. Although E211K (which is analogous to the E200K variant in humans that contributes to CJD) has been reported, it is not associated with typical BSE but rather an atypical form of this disease [10,25].

Susceptibility or resistance to TSE is also associated with variations in the non-coding region of *PRNP*, including the promoter and/or enhancer. Nucleotide changes in the non-coding region may affect mammalian PrP^C expression, which is also known to affect TSE susceptibility or resistance [33]. In cattle, strong associations between susceptibility to classical BSE and two insertion/deletion (indel) polymorphisms located in two regulatory regions of bovine *PRNP* have been identified [16,33]. These polymorphisms are a 23-bp indel at position −1594 and a 12-bp indel at position +300 (the positions of the polymorphisms are given with respect to the transcription start site in GenBank accession No. AJ298878); both are associated with promoter activity and bovine PrP^C expression levels *in vitro* [32]. Therefore, these polymorphisms can both seemingly influence BSE incubation times and susceptibility. The two indel polymorphisms and their frequencies have been previously identified and characterized in a number of bovine strains such as Holstein, Brown Swiss, Fleckvieh, and Japanese Black [9,16,24,33].

Hanwoo cattle [*Bos (B.) taurus coreanae*] were created by crossbreeding between *B. indicus* and *B. taurus*, and are now widely raised in Korea as a native strain [12]. To date, no BSE cases have been reported in Hanwoo. Genotypic descriptions of BSE-related genetic factors, such as SNPs or the number of octapeptide repeats in *PRNP*, have rarely been reported for Hanwoo, and the genetic frequencies and features related to BSE susceptibility have been observed in only a limited number of samples [14,15]. In the present study, we performed a detailed analysis of both sequence variations in the coding region of *PRNP* and prevalent indel polymorphisms in the non-coding region of *PRNP* using a large number of samples from Hanwoo cattle. Furthermore, we analyzed the genetic frequency and haplotype of gene variations associated with BSE susceptibility and compared them to those of other cattle breeds.

Materials and Methods

Animal samples and genomic DNA isolation
Muscle and hair samples for genomic DNA isolation were collected from 300 Hanwoo cattle in Korea. After collection, all samples were frozen until analysis. Genomic DNA was extracted both from 20 mg of muscle samples using a Puregene core kit (Qiagen, Germany) and from hair samples using a DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions.

Genotype analysis of protein coding region of *PRNP*
The PCR primers CDSF and CDSR were designed to amplify a 1,203-bp DNA fragment that included the entire protein-coding region of bovine *PRNP*. The primer sequences, melting temperature (Tm), and the annealing temperatures used for the amplification reaction are presented in Table 1. Each 40 μL of PCR mixture consisted of 100 ng of genomic DNA, 5 pmol of each primer, and 20 μL of PCR pre-mix (Superbio, Korea). The amplification for the entire coding region was performed using an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec. Finally, an extension step at 72°C for 5 min was performed. The amplified DNA fragments were analyzed by electrophoresis on a 1% agarose gel. Direct sequencing of the PCR products was performed using a 3730*xl* DNA analyzer (Applied

Table 1. Oligonucleotides used for PCR, sequencing, and genotyping in this study

	Primer name	Sequence	Tm (^{o}C)	Annealing temperature (^{o}C)	Product size (bp)
Protein-coding region	CDSF	5′-AGGTCAACTTTGTTAGAGAAG-3′	51.9	58	1,203
	CDSR	5′-TGAGCTAACGGGACTTGAATAAAC-3′	55.5		
Sequencing	PRNP-F1	5′-GCTGATGCCACTGCTATG-3′	50	54	
	PRNP-R1	5′-AATGAGACACCACCACTACG-3′	52		
23-bp indel	SH23F	5′-GTGCCAGCCATGTAAGTGACTGAA-3′	62.7	63	106 or 129
	SH23R	5′-AAGAGTTGGACAGGCACAATGGG-3′	62.4		
12-bp indel	PRNP-12F*	5′-TTACCCTCCTGGTTAGGAG-3′	56.7	54	91 or 103
	PRNP-12R*	5′-CTAGATTCCTACACACCAC-3′	54.5		

*The PRNP-12F and PRNP-12R primer sequences were previously reported by Sander *et al.* [33].

Biosystems, USA) according to the manufacturer's instructions. The primers PRNP-F1 (5´-GCTGATGCCA CTGCTATG-3´) and PRNP-R1 (5´-AATGAGACACCAC CACTACG-3´) were used for the sequencing reactions.

Genotype analysis of the 23-bp and 12-bp indel polymorphisms

SH23F and SH23R primers were used for detecting the 23-bp indel polymorphism at position -1594, and PRNP-12F and PRNP-12R primers were used to detect the 12-bp indel polymorphism at position +300 (the positions of the polymorphisms are given with respect to the transcription start site in GenBank accession No. AJ298878). The specific primer sequences, Tm, and annealing temperatures used for amplification are presented in Table 1. Based on a previous report by Sander *et al.* [33], the SH23F/SH23R primer pair was designed to amplify a 106- or 129-bp DNA fragment, and the PRNP-12F/PRNP-12R primer pair was designed to amplify a 91- or 103-bp DNA fragment. Each 20 μL of PCR mixture consisted of 50 ng of genomic DNA, 10 pmol of each primer, and 10 μL of PCR pre-mix (Superbio, Korea). DNA amplification was performed using an initial denaturation step at 95°C for 3 min followed by 30 cycles of denaturation at 94°C for 20 sec, annealing at 63°C for the 23-bp indel or 54°C for the 12-bp indel for 20 sec, and extension at 72°C for 20 sec. Finally, an extension step at 72°C for 3 min was performed. The genotypes of the indels were analyzed by electrophoresis on a 4% agarose gel (3 : 1, NuSieve-SeaKem; Lonza, Switzerland) based on the size of the PCR product.

Haplotype analysis

Unphased genotypes were tested for deviation from Hardy−Weinberg Equilibrium with the exact test using HapAnalyzer software (ver. 1.0.1; Korea National Institute of Health, Korea), which is a minimum haplotype analysis system for association studies, with a cutoff of 0.01 as previously described [17].

Statistical analysis

Allele, genotype, and haplotype frequencies were calculated using SPSS Statistics software (ver. 17.0; SPSS, USA). The associations between the allele, genotype, and haplotype frequencies were analyzed by cross-tabulation using Chi-square (for 2 × 2 tables) and Fisher's exact tests.

Results

Variations in protein coding region of *PRNP*

We assessed the protein-coding region sequences of *PRNP* in 300 Hanwoo cattle and discovered a total of six SNPs. The genotype and allele frequencies of these six SNPs are shown in Table 2. Four of the variations (G234A, C555T,

Table 2. Single nucleotide changes in the coding region of the prion protein gene in Hanwoo cattle

| Nucleotide	Amino acid	Genotype frequency*			Allele frequency (%)	
234	78Q	G/G	G/A	A/A	G	A
		106 (35.3)	150 (50.0)	44 (14.7)	60.3	39.7
467	Y156C	A/A	A/G	G/G	A	G
		299 (99.7)	1 (0.3)	0 (0.0)	99.8	0.2
555	185N	C/C	C/T	T/T	C	T
		285 (95.0)	15 (5.0)	0 (0.0)	97.5	2.5
576	192N	C/C	C/T	T/T	C	T
		200 (66.7)	96 (32.0)	4 (1.3)	82.7	17.3
630	210T	C/C	C/T	T/T	C	T
		285 (95.0)	15 (5.0)	0 (0.0)	97.5	2.5
669	Q223H	A	A/C	C/C	A	C
		299 (99.7)	1 (0.3)	0 (0.0)	99.8	0.2

*Values represent the number (%).

C576T, and C630T) were synonymous polymorphisms that have been previously described by Brunelle *et al.* [4]. None of these particular substitutions resulted in an amino acid replacement in the corresponding protein sequence. Two other SNPs, A467G and A669C, were non-synonymous. That is, the point mutation (G for A) at nucleotide 467 encoded Cys (TGT) instead of Tyr (TAT) at residue 156, and the point mutation (C for A) at nucleotide 669 encoded His (CAC) instead of Gln (CAA) at residue 223 (Fig. 1). However, each of these mutations was found only once in this study.

We also identified that both the amino acid sequence and number of the octapeptide repeats were highly conserved. The numbers of octapeptide repeats in the *PRNP* SNPs are shown in Table 3. Furthermore, we did not find any sequence alteration or variation in the number of octapeptide repeats in the Hanwoo *PRNP* gene in our study. Table 3 includes a comparison of the number of octapeptide repeats in several breeds of cattle including German, Japanese Holstein, Japanese Black, and Slovak [11,16,24,33]. All of the Hanwoo animals examined in this study had six octapeptide repeats homozygously in the *PRNP* gene.

Variations in frequencies of the 23-bp and 12-bp indel polymorphisms

We performed a PCR-based genetic analysis of the 23-bp and 12-bp indel polymorphisms in the Hanwoo samples. Specifically, we assessed allele frequency, genotype frequency, and the haplotype data of the 23-bp and 12-bp indel polymorphisms in the Hanwoo cattle. We also analyzed data of other cattle breeds from previous reports [16,24] and compared these with our own results from the

PRNP on chromosome 13
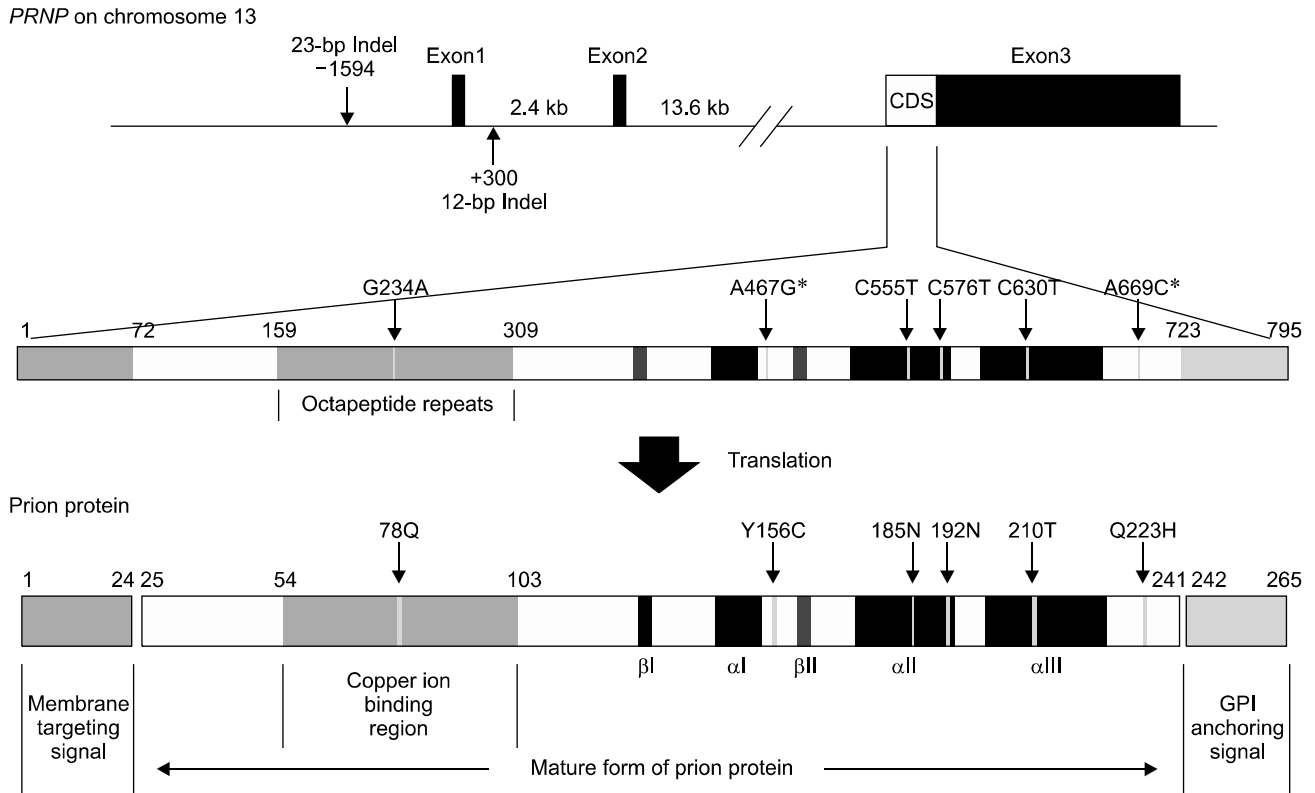
Fig. 1. Schematic diagram of the genetic variations of *PRNP* described in this study. Polymorphisms of the 23-bp indel at −1594 and 12-bp indel at +300 are in the non-coding region. Six single nucleotide changes, including four synonymous SNPs and two non-synonymous mutations (indicated with an asterisk), are found in the coding region of *PRNP* in this study. The functional roles of each domain are also shown. The positions of the polymorphisms are relative to the transcription start site in GenBank accession No. AJ298878.

present study.

Results of the Chi-square (for 2 × 2 tables) and Fisher's exact tests revealed significant associations between each polymorphism of the 23-bp and 12-bp indels, and BSE occurrence in the reported populations (Table 4). When comparing the data from the various groups of healthy animals, we found that Hanwoo cattle had a lower deletion frequency (58.0%) of the 23-bp indel polymorphism than all other breeds (70.8% in UK Holstein, 61.7% in German Holstein, 68.0% in German Fleckvieh, 78.8% in Japanese Holstein, and 59.1% in Japanese Black) except for German Brown (34.5%). Additionally, Hanwoo had a lower deletion frequency (54.8%) of the 12-bp indel polymorphism than the other breeds (63.0% in UK Holstein, 62.0% in German Fleckvieh, 73.8% in Japanese Holstein, and 56.7% in Japanese Black) except for German Holstein (53.1%) and German Brown (14.4%).

Upon comparing the results from the healthy and BSE-affected groups, we found that both the 23-bp and 12-bp deletion allele frequencies of the BSE-affected group were significantly higher than those of the healthy UK Holstein, German Holstein, and German Brown cattle.

Table 3. Number of octapeptide repeats in the *PRNP* gene

Breed	Geno type*	BSE-affected		Healthy	
		Number	Frequency (%)	Number	Frequency (%)
Hanwoo	6/6	−	−	300	100
	6/5	−	−	0	0
	5/5	−	−	0	0
German[†]	6/6	39	91	43	90
	6/5	4	9	5	10
	5/5	0	0	0	0
Japanese Holstein[‡]	6/6	6	100	765	88
	6/5	0	0	94	11
	5/5	0	0	4	1
Japanese Black[‡]	6/6	−	−	173	93
	6/5	−	−	13	7
	5/5	−	−	0	0
Slovak[§]	6/6	22	92	262	87
	6/5	2	8	36	12
	5/5	0	0	3	1

*The number of octapeptide repeats reported by [†]Sander *et al.* [33], [‡]Nakamitsu *et al.* [24], and [§]Hresko *et al.* [11].

Table 4. Allele frequencies and statistical comparisons for the 23-bp and 12-bp indels

| | | 23-bp indel | | | | | | | 12-bp indel | | | | | | |
| | | BSE-affected | | | Healthy | | | | BSE-affected | | | Healthy | | | |
Breed	Allele	Number	Frequency (%)	$\chi^{2\dagger}$ (p)	Number	Frequency (%)	$\chi^{2\dagger}$ (p)	$\chi^{2\ddagger}$ (p)	Number	Frequency (%)	$\chi^{2\dagger}$ (p)	Number	Frequency (%)	$\chi^{2\dagger}$ (p)	$\chi^{2\ddagger}$ (p)
Hanwoo	del	–	–	–	348	58.0	–	–	–	–	–	329	54.8	–	–
	ins	–	–		252	42.0			–	–		271	45.2		
UK Holstein §	del	551	75.9	48.186* (<0.001)	391	70.8	20.589* (<0.001)	4.146* (0.047)	505	72.1	42.093* (<0.001)	340	63.0	7.747* (0.006)	11.834* (0.001)
	ins	175	24.1		161	29.2			195	27.9		200	37.0		
German Holstein §	del	175	68.9	8.928* (0.003)	386	61.7	1.709 (0.200)	4.094* (0.045)	157	62.8	4.574* (0.033)	328	53.1	0.379 (0.565)	6.829* (0.010)
	ins	79	31.1		240	38.3			93	37.2		290	46.9		
German Brown §	del	46	53.5	0.626 (0.484)	60	34.5	29.928* (<0.001)	8.609* (0.005)	28	32.6	14.954* (<0.001)	26	14.4	91.08* (<0.001)	11.801* (0.001)
	ins	40	46.5		114	65.5			58	67.4		154	85.6		
German Fleckvieh §	del	156	73.6	16.161* (<0.001)	185	68.0	7.899* (0.005)	1.776 (0.193)	145	68.4	11.859* (0.001)	170	62.0	3.992* (0.047)	2.115 (0.152)
	ins	56	26.4		87	32.0			67	31.6		104	38.0		
Japanese Holstein ‖	del	10	83.3	3.110 (0.136)	438	78.8	57.243* (<0.001)	0.146 (1.000)	10	83.3	3.867 (0.075)	428	73.8	46.099* (<0.001)	0.556 (0.740)
	ins	2	16.7		118	21.2			2	16.7		152	26.2		
Japanese Black ‖	del	–	–	–	220	59.1	0.123 (0.738)	–	–	–	–	211	56.7	0.331 (0.595)	–
	ins	–	–		152	40.9			–	–		161	43.3		

Allele frequencies were compared using Fisher's exact and Chi-square tests. *Cases in which a significant difference (p < 0.05) was observed. †Chi-square test comparing experimental group and Hanwoo cattle (p, p-values from Fisher's exact tests). ‡Chi-square test comparing healthy and BSE-affected groups (p, p-values from Fisher's exact tests). These data were reported by §Juling et al. [16] and ‖ Nakamitsu et al. [24].

Table 5. Comparison of genotype frequencies for the 23-bp and 12-bp indels

| | | 23-bp indel | | | | | | | 12-bp indel | | | | | | |
| | | BSE-affected | | | Healthy | | | | BSE-affected | | | Healthy | | | |
Breed	Genotype	Number	Frequency (%)	$\chi^{2\dagger}$ (p)	Number	Frequency (%)	$\chi^{2\dagger}$ (p)	$\chi^{2\ddagger}$ (p)	Number	Frequency(%)	$\chi^{2\dagger}$ (p)	Number	Frequency (%)	$\chi^{2\dagger}$ (p)	$\chi^{2\ddagger}$ (p)
Hanwoo	del/del	–	–	–	89	29.6	–	–	–	–	–	85	28.3	–	–
	del/ins	–	–		170	56.7			–	–		159	53.0		
	ins/ins	–	–		41	13.7			–	–		56	18.7		
UK Holstein §	del/del	201	55.4	53.65* (<0.001)	128	46.4	24.59* (<0.001)	5.138 (0.075)	173	49.4	45.96* (<0.001)	100	37.0	8.279* (0.013)	13.63* (0.001)
	del/ins	149	41.0		135	48.9			159	45.4		140	51.9		
	ins/ins	13	3.6		13	4.7			18	5.1		30	11.1		
German Holstein §	del/del	58	45.7	10.88* (0.005)	119	38.0	5.863 (0.055)	4.632 (0.097)	50	40.0	4.917 (0.092)	87	28.2	1.132 (0.562)	6.883* (0.033)
	del/ins	59	46.5		148	47.0			57	45.6		154	49.8		
	ins/ins	10	7.9		46	14.7			18	14.4		68	22.0		
German Brown §	del/del	9	20.9	1.472 (0.497)	12	13.8	41.15* (<0.001)	12.13* (0.001)	3	7.0	16.09* (<0.001)	3	3.3	101.8* (<0.001)	14.41* (0.001)
	del/ins	28	65.1		36	41.4			22	51.2		20	22.2		
	ins/ins	6	14.0		39	44.8			18	41.9		67	74.4		
German Fleckvieh §	del/del	57	53.8	20.32* (<0.001)	63	46.3	11.44* (0.004)	1.803 (0.411)	48	45.3	12.64* (0.002)	54	39.4	5.342 (0.075)	2.766 (0.258)
	del/ins	42	39.6		59	43.4			49	46.2		62	45.3		
	ins/ins	7	6.6		14	10.3			9	8.5		21	15.3		
Japanese Holstein ‖	del/del	4	66.7	4.039 (0.179)	174	62.6	64.6* (<0.001)	0.319 (—)	4	66.7	4.524 (0.156)	159	54.8	47.12* (<0.001)	0.617 (1.000)
	del/ins	2	33.3		90	32.4			2	33.3		110	37.9		
	ins/ins	0	0.0		14	5.0			0	0.0		21	7.3		
Japanese Black ‖	del/del	–	–	–	53	28.5	1.582 (0.454)	–	–	–	–	49	26.3	3.715 (0.163)	–
	del/ins	–	–		114	61.3			–	–		113	60.8		
	ins/ins	–	–		19	10.2			–	–		24	12.9		

Genotype frequencies were compared using Fisher's exact and Chi-square tests. *Cases in which a significant difference (p < 0.05) was observed. †Chi-square test comparing experimental group and Hanwoo cattle (p, p-values from Fisher's exact tests). ‡Chi-square test comparing healthy and BSE-affected groups (p, p-values from Fisher's exact tests). These data were reported by §Juling et al. [16] and ‖ Nakamitsu et al. [24].

Among UK Holstein animals, the 23-bp deletion allele frequencies for the BSE-affected and healthy groups were 75.9 and 70.8% ($p < 0.05$), respectively, while the 12-bp deletion allele frequencies were 72.1 and 63.0% ($p < 0.01$), respectively. Among German Holsteins, the 23-bp deletion allele frequencies for the BSE-affected and healthy groups were 68.9 and 61.7% ($p < 0.05$), respectively, while the 12-bp deletion allele frequencies were 62.8 and 53.1% ($p < 0.05$), respectively. For German Brown, the 23-bp deletion allele frequencies for the BSE-affected and healthy groups were 53.5 and 34.5% ($p < 0.01$), respectively, while the 12-bp deletion allele frequencies were 32.6 and 26.0% ($p < 0.01$), respectively.

In addition, the deletion frequencies of the BSE-affected groups in the other two breeds (German Fleckvieh and Japanese Holstein) were higher than those of the healthy groups although this difference was not statistically significant. For German Fleckvieh, the 23-bp deletion allele frequencies for the BSE-affected and healthy groups were 73.6 and 68.0%, respectively, while the 12-bp deletion allele frequencies were 68.4 and 62.0%, respectively. In Japanese Holstein, the 23-bp deletion allele frequencies for the BSE-affected and healthy groups were 83.3 and 78.8%, respectively, and the 12-bp deletion allele frequencies were 83.3 and 73.8%, respectively.

BSE-affected animals of almost all breeds had significantly higher deletion frequencies than the healthy groups for both the 23-bp and 12-bp indel polymorphisms.

Hanwoo cattle had lower deletion frequencies for both the 23-bp and 12-bp indel polymorphisms compared to other breeds. Thus, differences in the deletion frequencies for the 23-bp and 12-bp indels between Hanwoo cattle and BSE-affected animals of other breeds were more significant (for the 23-bp indel: $p < 0.001$ for UK Holstein, $p < 0.01$ for German Holstein, and $p < 0.001$ for German Fleckvieh; for the 12-bp indel: $p < 0.001$ for UK Holstein, $p < 0.05$ for German Holstein, $p < 0.001$ for German Brown, and $p < 0.01$ for German Fleckvieh).

Genotype frequencies of the 23-bp and 12-bp indel polymorphisms are shown in Table 5. For the 23-bp indel polymorphism in the healthy groups of various breeds, the del/del frequency for Hanwoo cattle was 29.6%. This was significantly lower than those of all other breeds (46.4% for UK Holstein, 38.0% for German Holstein, 46.3% for German Fleckvieh, and 62.6% for Japanese Holstein) except for German Brown (13.8%) and Japanese Black (28.5%). For the 12-bp indel polymorphism in the healthy animals, the del/del frequency for Hanwoo cattle was 28.3%. This was significantly lower than those of UK Holstein (37.0%), German Fleckvieh (39.4%), and Japanese Holstein (54.8%) but not German Holstein (28.2%), German Brown (3.3%), or Japanese Black (26.3%).

In general, BSE-affected cattle had a relatively higher del/del frequency for both the 23-bp and 12-bp indel polymorphisms than the healthy animals of each breed.

Table 6. Comparison of haplotype frequencies for the 23-bp and 12-bp indels

	Haplotype	BSE-affected			Healthy			$\chi^{2\ddagger}$ (p)
		Number	Frequency	$\chi^{2\dagger}$ (p)	Number	Frequency	$\chi^{2\dagger}$ (p)	
Hanwoo	23del/12del	—	—	—	329	55.0	—	—
	23del/12ins	—	—		19	3.0		
	23ins/12ins	—	—		252	42.0		
UK Holstein[§]	23del/12del	506	71.7	47.149*	343	62.8	29.849*	14.604*
	23del/12ins	29	4.1	(<0.001)	44	8.1	(<0.001)	(0.001)
	23ins/12ins	171	24.2		159	29.1		
German Holstein[§]	23del/12del	162	62.2	14.675*	327	51.3	20.139*	7.580*
	23del/12ins	17	7.2	(0.001)	56	9.1	(<0.001)	(0.023)
	23ins/12ins	77	30.2		232	39.1		
German Brown[§]	23del/12del	27	32.5	55.105*	26	14.4	121.545*	12.756*
	23del/12ins	18	22.5	(<0.001)	35	19.4	(<0.001)	(0.002)
	23ins/12ins	39	45.0		119	66.1		
German Fleckvieh[§]	23del/12del	147	68.1	16.297*	171	62.0	8.920*	52.061*
	23del/12ins	11	5.1	(<0.001)	15	5.4	(0.012)	(<0.001)
	23ins/12ins	58	26.8		90	32.6		

Haplotype frequencies were compared using Fisher's exact and Chi-square tests. *Cases in which a significant difference ($p < 0.05$) was observed. [†]Chi-square test comparing experimental group and Hanwoo cattle (*p, p*-values for Fisher's exact tests). [‡]Chi-square test comparing the healthy and BSE-affected groups (*p, p*-values of Fisher's exact tests). [§]These data were reported by Juling *et al.* [16].

However, differences in the genotype frequencies for the 23-bp indel between the BSE-affected and healthy groups were statistically significant for only German Brown cattle ($p < 0.01$). Moreover, differences in the genotype frequencies for the 12-bp indel between the BSE-affected and healthy groups were significant among UK Holstein ($p < 0.01$), German Holstein ($p < 0.05$), and German Brown ($p < 0.01$) cattle.

To estimate the combined effect of both the 23-bp and 12-bp indel polymorphisms, we performed a haplotype analysis. The results of this analysis are summarized in Table 6. We observed strong linkage disequilibrium in all populations. The major haplotypes observed were 23 del/12 del, 23 ins/12 ins, and 23 del/12 ins. In contrast, 23 ins/12 del was a minor haplotype (less than 1%) and was thus excluded from further analyses. In all breeds, the 23 del/12 del haplotype frequencies of the BSE-affected groups were higher than those of the healthy groups. On the other hand, the 23 ins/12 ins haplotype frequencies of the BSE-affected groups were lower than those of the healthy groups. The frequencies of the Hanwoo cattle were significantly different ($p < 0.05$) than those of all other populations.

Haplotype analysis

We analyzed the bovine *PRNP* haplotype structures by evaluating a combination of SNPs, the number of octapeptide repeats in the prion protein-coding region, and the 23-bp and 12-bp indel polymorphisms in the non-coding region. For clarity, we identified haplotypes with a frequency above 0.01 and found a total of 19 in Hanwoo, *B. taurus*, and *B. indicus* (Table 7). Hanwoo and *B. taurus* had similar patterns of these haplotype frequencies. In particular, haplotypes 3 and 13 were the most frequent in both Hanwoo and *B. taurus* (72 and 78% among these populations, respectively) whereas these accounted for only 17% of *B. indicus* haplotypes. Furthermore, haplotype 5 accounted for 17% of the haplotypes among the Hanwoo population, but it was rarely observed in either *B. taurus* or *B. indicus*.

Discussion

It has been established that polymorphisms in the *PRNP* gene are associated with susceptibility to prion diseases in humans, sheep, and mice [1,22,38]. Many studies have also explored the relationships between *PRNP* polymorphisms and BSE susceptibility in cattle

Table 7. Haplotypes and their respective frequencies for Hanwoo, *Bos* (*B.*) *taurus,* and *B. indicus*

	23-bp	12-bp	69	75	108	126	oct	234	339	361	555	576	630	675	678	Frequency (%)		
																*B. indicus**	Hanwoo	*B. taurus**
1	D	D	C	G	T	A	6	A	C	G	C	C	C	C	T	–	< 1	6
2	D	D	C	G	T	A	6	G	T	G	C	C	C	C	T	1	–	2
3	D	D	C	G	T	A	6	G	C	G	C	C	C	C	T	12	37	40
4	D	D	C	G	T	A	6	G	C	G	C	T	C	C	T	–	–	4
5	D	D	C	G	T	A	6	G	C	G	T	C	C	C	T	–	17	–
6	D	I	C	G	T	A	5	–	C	G	C	C	C	C	T	–	–	3
7	D	I	C	G	T	A	5	–	C	G	C	C	C	C	C	2	–	–
8	D	I	C	G	T	A	6	A	C	G	T	C	C	C	T	10	–	–
9	D	I	C	G	T	A	6	A	C	G	T	C	T	C	T	14	–	–
10	D	I	C	G	T	A	6	A	C	G	C	T	C	C	T	–	2	–
11	D	I	C	G	T	A	6	G	C	G	C	C	C	C	T	30	–	1
12	D	I	T	G	T	A	6	G	C	G	C	C	C	C	T	17	–	< 1
13	I	I	C	G	T	A	6	A	C	G	C	C	C	C	T	5	35	38
14	I	I	C	G	T	A	6	A	C	G	C	C	T	C	T	–	2	–
15	I	I	C	G	T	A	6	G	T	G	C	C	C	C	T	–	–	1
16	I	I	C	G	T	A	6	G	C	G	C	C	C	C	T	–	5	2
17	I	I	C	G	A	G	6	A	C	A	C	C	C	T	C	3	–	–
18	I	I	C	A	A	G	6	G	C	A	C	C	C	C	C	3	–	–
19	I	I	T	G	T	A	6	G	C	G	C	C	C	C	T	–	–	< 1
		Total haplotype frequency														97	98	97

The haplotype positions are the 23-bp and 12-bp insertions (I) or deletions (D), the nucleotide position in the *PRNP* coding sequence, and the number of octapeptide repeats (oct). *These data were reported by Brunelle *et al.* [4].

[16,26,33]. The majority of these studies have been focused on investigating both the protein-coding and non-coding regions of the *PRNP* gene to search for polymorphisms that affect BSE susceptibility. It has been demonstrated that non-synonymous alterations in the protein-coding region of *PRNP* can cause structural changes whereas nucleotide changes in the regulatory region including the promoter can modulate *PRNP* expression [33]. Of note, these variations may influence BSE susceptibility or resistance. To date, there are no known polymorphisms associated with susceptibility to BSE in cattle with the exception of the E211K variant, an analogue of the E200K variant in humans that affects CJD [10]. However, E211K has been reported in only two animals, one of which had BSE; this case of BSE was atypical rather than typical [10,25].

In the present study, we identified four variations (G234A, C555T, C576T, and C630T) in the *PRNP* coding region that were synonymous polymorphisms. In previous studies of the *PRNP* gene in Hanwoo cattle, only a few genetic variations in the *PRNP* coding region were reported. Jeong *et al.* [15] analyzed the open reading frame (ORF) region of the *PRNP* gene in 120 Hanwoo cattle and found two polymorphisms: the third position of codon 78 (G234A) and the third position of codon 192 (C576T). However, these authors did not find any variation in the octapeptide repeat. These polymorphisms (G234A and C576T), which were also found in our present study, are commonly found in both *B. taurus* and *B. indicus* [4]. However, C555T and C630T detected in our study have been previously found only in *B. indicus* [4]. Two SNPs (A467G and A669C) identified in our study are point mutations because their minor allele frequencies were especially low (less than 0.01) in the experimental population. Thus, our findings indicate that the polymorphisms found in this study cannot induce structural changes in the prion protein.

The octapeptide repeat region near the N-terminal portion of the prion protein has a binding affinity to divalent cations, and may affect protein structure and function [7]. It has been established that this protein commonly have five or six repeats [24]. The presence of extra repeats is correlated with increased TSE susceptibility as has been previously observed in transgenic mice expressing bovine PrP^C with four extra octapeptide repeats as well as humans with nine octapeptide repeat insertion in the *PRNP* gene [6,19]. It has also been shown that Brown Swiss cattle expressing seven octapeptide repeats are more susceptible to BSE than animals of other breeds [4,34,36]. Collectively, these findings indicate that bovine PrP^C containing extra octapeptide repeats may enhance susceptibility to BSE. All Hanwoo animals sampled in the present study had six octapeptide repeats, which was different from animals of other breeds with either five or six octapeptide repeats. Thus, Hanwoo may have a relatively lower risk of being infected with BSE based on their conserved number of octapeptide repeats encoded in the *PRNP* gene.

A tentative association between *PRNP* promoter polymorphisms, including the 23-bp and 12-bp indels, and BSE susceptibility has been previously reported in German cattle [33]. This study revealed that the frequencies of these two polymorphisms are significantly different between BSE-affected and healthy cattle populations in terms of both allele and genotype frequencies. These polymorphisms are thought to be responsible for differences in BSE incubation time and/or susceptibility, but are not involved in resistance to atypical BSE [5,33]. Results of previous reporter gene assays have suggested that both the 23-bp and 12-bp indel polymorphisms are involved in modulating the expression of bovine *PRNP* [32]. It has been established that the 23-bp indel polymorphism within the 5′-flanking region is responsible for a binding site for the RP58 repressor protein, which is expressed mainly in the brain, and the 12-bp indel polymorphism within intron 1 is responsible for a binding site for the SP1 transcription factor involved in the regulation of numerous promoters [13,32].

Recently, it has been reported that polymorphic differences could be the reason for changes in *PRNP* expression levels based on the significant correlation between 23-bp indel polymorphisms and *PRNP* mRNA expression in two Japanese cattle breeds [23]. Thus, the 23-bp deletion can restrict the access of the RP58 repressor and induce overexpression of the prion protein, thereby increasing BSE susceptibility, whereas the 12-bp deletion can decrease *PRNP* promoter activity and reduce expression of the prion protein [32]. However, the allele frequency of the 12-bp deletion has been shown to be higher in BSE-affected animals than in healthy ones [33]. These apparently contradictory results can be explained by the following logic. First, there is strong linkage between the 23-bp and 12-bp indels. In other words, the rate of crossover between the 23-bp and 12-bp indels is quite low, which leads to linkage disequilibrium. In our haplotype analysis of combinations including both 23-bp and 12-bp indels, we found that the 23 del/12 del and 23 ins/12 ins haplotypes were common (more than 80%) while the 23 del/12 ins frequency was relatively low. Very few cases of the 23 ins/12 del haplotype, which were considered statistically negligible (less than 1%), were observed in the UK Holstein, German Holstein, German Brown, German Fleckvieh, or Hanwoo cattle. Second, the 23-bp deletion seems to have a more crucial role in BSE susceptibility than the 12-bp insertion. According to the reporter gene assays previously performed by Sander *et al.* [32], the 23 ins/12 ins haplotype showed weaker promoter activity than the 23 del/12 del haplotype although the promoter activity of the 12-bp insertion was stronger than that of the 23-bp deletion. These results can be explained by the previous finding that, with the 23 ins/12 ins haplotype, SP1 binding on the 12-bp

insertion can be repressed by RP58 binding on the 23-bp insertion [20]. Promoter activity associated with the 12-bp indel is thus dependent upon the 23-bp indel. Therefore, although the 12-bp deletion has a negative effect on BSE susceptibility according to previous *in vitro* experiments, a higher frequency of the 12-bp deletion with the 23-bp deletion can be found in BSE-affected animals compared to healthy controls.

In China, the frequency distribution of genotypes, alleles, and haplotypes for these 12-bp and 23-bp indel polymorphisms of 349 native Chinese cattle were recently investigated [39]. Relatively low frequencies of the 12-bp deletion allele and 23 del/12 del haplotype, which have been suggested to affect BSE susceptibility, were observed. Another Chinese group identified SNPs in exon 3, a 23-bp indel in the promoter region, and a 12-bp indel in intron 1 in the *PRNP* gene from two Chinese indigenous cattle breeds of northeast China [30]. They found that there three different genotypes exist among both Yanbian and Chinese Red Steppes breeds in the 23-bp indel and 12-bp indel loci, and that 23del/12del is the main haplotype accounting for more than 50% of all Yanbian and Chinese Red Steppes cattle.

Since there were no BSE cases in the Hanwoo population, we could not compare differences in the 23-bp and 12-bp indel polymorphisms between healthy and BSE-affected Hanwoo cattle. Therefore, we compared our data for the Hanwoo cattle with data for other breeds from previous studies by Juling *et al.* [16] and Nakamitsu *et al.* [24]. We found that the deletion allele frequencies of 23-bp and 12-bp indel polymorphisms were lower for Hanwoo than those for the other breeds except for German Brown. In addition, the difference in allele frequencies between Hanwoo cattle and BSE-affected animals was significant ($p < 0.05$). According to the haplotype analysis, Hanwoo had a lower 23 del/12 del frequency than the other breeds except for German Brown (both BSE-affected and healthy) and German Holstein (healthy). These data indicate that the Hanwoo population is likely to have a lesser risk for BSE compared to other cattle breeds. Kim *et al.* [18] also surveyed the allelic, genotypic, and haplotypic distribution of the 23-bp and 12-bp indel *PRNP* polymorphisms from Korean cattle. This study revealed that the allelic frequencies for the 23-bp indel were 44.3% (insertion) and 57.7% (deletion), whereas they were 44.4% (insertion) and 55.6% (deletion) for the 12-bp indel [18]. These particular results are similar to ones from our own investigation. However, the genotypes examined in the Kim *et al.* [18] study had more homologous patterns with a lower del/ins frequency than those in our investigation. Allelic and genotypic frequencies for the 23-bp and 12-bp indels similar to those found by Kim *et al.* [18] have also been observed in another previous study of Hanwoo cattle.

In conclusion, our results demonstrated that Hanwoo cattle do not have any unique alterations that cause structural changes in the prion protein associated with BSE susceptibility. In addition, we found that all of the Hanwoo cattle included in this study had six octapeptide repeats, which are typically found in cattle and do not influence BSE susceptibility. For both the 23-bp and 12-bp indel polymorphisms, Hanwoo exhibited low levels of deletion frequencies. Since these 23-bp and 12-bp deletion alleles are associated with a higher susceptibility to BSE as reported in other breeds [33], it is likely that Hanwoo cattle are less susceptible to BSE than those of other breeds based on their lower deletion frequency in the 23-bp and 12-bp indel polymorphisms. To further clarify the relationship between genetic variations and functional alterations of the *PRNP* gene in Hanwoo cattle, further functional analyses should be performed.

Acknowledgments

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